Accepted refereed manuscript of: Goncalves RA, Navarro-Guillen C, Gilannejad N, Dias J, Schatzmayr D, Bichl G, Czabany T, Moyano FJ, Rema P, Yufera M, MacKenzie S & Martinez-Rodriguez G (2018) Impact of deoxynivalenol on rainbow trout: Growth performance, digestibility, key gene expression regulation and metabolism, *Aquaculture*, 490, pp. 362-372.

DOI: https://doi.org/10.1016/j.aquaculture.2018.03.001

Impact of deoxynivalenol on rainbow trout: Growth performance, digestibility, key gene expression regulation and metabolisation

Running title: Impact of deoxynivalenol on rainbow trout

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Abstract

The impact of deoxynivalenol (DON) on rainbow trout, *Onchorynchus mykiss*, is mainly characterised by impaired growth performance and reduced feed intake, usually with the total absence of any visible clinical signs. Despite the high concentrations of DON in the present study (up to 11,412 ± 1,141 µg/kg), no clinical signs (except anorexia at the higher DON dosage) were observed, which confirms the difficulties of diagnosing DON ingestion. Compared to the control group, the proteolytic enzyme activities (pepsin, trypsin and chymotrypsin) in trout were altered by DON ingestion. However, it was not clear if the observed impact on digestive enzymes was due to the direct action of DON, or a consequence of the lower feed intake determined for DON-treated animals. The impact of DON on the abundance of specific measured mRNA transcripts was unexpected with higher expression levels for insulin-like growth factors*, igf1* and *igf2*, which are directly related to elevated insulin levels in plasma. This can also in part be influenced by the trypsin activity and by *npy*, given its higher mRNA expression levels. The apparent digestibility of dry matter, protein and energy was not affected by dietary levels of DON, however, nutrient retention, protein, fat and energy retention were significantly affected in animals fed DON. Adenylate cyclase-activating polypeptide (PACAP) expression seems to play an important role in controlling feed intake in DON fed trout. In the present study, we have shown for the first time that DON is metabolised to DON-3-sulfate in trout. DON-3-sulfate is much less toxic than DON, which helps to explain the lack of clinical signs in fish fed DON. Being a novel metabolite identified in trout makes it a potential biomarker of DON exposure. Suppression of appetite due to DON contamination in feeds might be a defence mechanism in order to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON.

Keywords: *Fusarium* mycotoxins, *Oncorhynchus mykiss,* PACAP, DON-3-sulfate, biomarker

Abbreviations

ZFIN Zebrafish Nomenclature Guidelines have been followed for all fish genes and proteins described in this manuscript

(https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines#ZFINZebrafishNomenclatureGuidelines-1).

Gene abbreviations:

ef1a - elongation factor 1 alpha
actb - beta actin
star - steroidogenic acute regulatory protein
Igf1 - insulin-like growth factor 1
Igf2 - insulin-like growth factor 2
crf1 - corticotropin releasing factor precursor 1
crf2 - corticotropin releasing factor precursor 2
crfbp - corticotropin releasing factor binding protein precursor
npy - neuropeptide Y precursor
adcyap1a - growth hormone-releasing hormone/pituitary adenylate cyclase-activating polypeptide (PACAP)
lep - leptin
sst2 - somatostatin-2 precursor
chia - gastric chitinase
pga - pepsinogen
lpl - lipoprotein lipase
ghrl - ghrelin/obestatin preprohormone
cel1 - carboxyl ester lipase 1
cel2 - carboxyl ester lipase 2
cckt - cholecystokinin (Tyrosine)
cckn - cholecystokinin (Asparagine)
cckl - cholecystokinin (Leucine)
amy2a1 - pancreatic alpha amylase
atp4a - ATPase H+/K+ transporting alpha subunit
crtl - chymotrypsinogen-like precursor
try1 - trypsinogen 1 precursor
try2 - trypsinogen 2 precursor
try3 - trypsinogen 3 precursor

1. Introduction

Mycotoxins, toxic secondary metabolites produced by moulds (Hussein, Brasel, 2001), can be produced on agricultural commodities before and/or after harvest, during transportation or storage. Mycotoxins are a significant problem worldwide causing adverse health outcomes when consumed by humans and animals (Zain, 2011) and are responsible for significant economic losses worldwide due to condemned agricultural products (CAST, 2003; Shane, Eaton, 1994; Vasanthi, Bhat, 1998). The presence of mycotoxins in aquaculture are not novel. The first report of mortality due to mycotoxicosis in aquaculture was in the early 1960s, where in the United States, hatchery-reared rainbow trout (*Oncorhynchus mykiss*) were fed cottonseed meal contaminated with aflatoxins (Kumar et al., 2013; Wolf, Jackson, 1963).

In recent years, the awareness of mycotoxin-related issues in the industry has been raised again, mainly due to the increasing inclusion levels of plant meals in aquafeeds (Anater et al., 2016; Gonçalves et al., 2016; Gonçalves et al., 2017; Hooft, Bureau, 2017; Hooft et al., 2011). Feed manufacturers and producers realise the importance of mycotoxins and their potential negative effects on production. However, there are still two major constraints entrenched in the aquaculture industry that make it difficult to develop an effective mycotoxin management plan for the sector.

The first constraint is that the majority of mycotoxin issues stem from poor storage conditions. Poor storage conditions can lead to the growth of *Aspergillus spp*. and *Penicillium spp*., which can ultimately lead to the production of AF and ochratoxin A (OTA). However, in countries where climate conditions are favourable to the growth of *Aspergillus spp*. and *Penicillium spp*. fungi, optimal storage conditions should prevent the contamination of raw materials and finished feeds with AF or OTA. However, the same is not true for *Fusarium spp*., which on the contrary to *Aspergillus spp*. and *Penicillium spp*., are more likely to grow in the crops pre-harvest. The *Fusarium* mycotoxins (Type B and A trichothecenes and fumonisins) are reasonably stable to processing conditions (Cheli et al., 2013). Therefore, these mycotoxins are not destroyed during raw material processing or aquafeed manufacturing, and will consequently be present in the finished feed. This may lead to potential harm to fish and shrimps, dependent upon concentration and co-occurrence. We have previously reported that soybean meal, wheat, wheat bran, maize, corn gluten meal, rapeseed/canola meal and rice bran in samples from Asian were mostly contaminated with

Fusarium mycotoxins (zearalenone, ZEA; deoxynivalenol, DON; and fumonisin B_1, FB) (Gonçalves, Naehrer et al., 2016; Gonçalves, Schatzmayr et al., 2017). Thus, finished feed samples were mainly contaminated with *Fusarium* mycotoxins, reflecting the use of plant meals in the finished feeds and not resulting from poor storage conditions. Moreover, the presence of secondary metabolites of *Fusarium* spp. are expected to increase as a response to climate change (Miraglia et al., 2009; Paterson, Lima, 2010; Paterson, Lima, 2011) which will likely further impact upon the global aquaculture industry. Among the metabolites produced by the *Fusarium* genus, DON is reported as the main mycotoxin found especially in small grain cereals (Rodrigues, Naehrer, 2012; Simsek et al., 2013). Despite the fact that the toxic effects and toxicokinetics of DON, a mycotoxin commonly known as "vomitoxin" as it causes vomiting in livestock, is well described including clinical symptoms for land farmed animals (Pestka, 2007), very little is known for aquatic animals.

The second constraint for mycotoxin research in aquaculture is the lack of any validated clinical symptoms in fish and shrimps when they are fed mycotoxins. Several reports describe some clinical signs for the most common mycotoxins (see review from Anater, Manyes et al. (2016)), however they are generalised and could be attributed to any diverse pathologies or challenges e.g. anti-nutrition factors or lectins in the diet (Hart et al., 2010). Two notable exceptions are aflatoxicosis (yellowing of the body surface, (Deng et al., 2010) and ingestion of FB (alteration of the sphinganine to sphingosine ratio, (Tuan et al., 2003). Most reported clinical manifestations due to mycotoxin ingestion are related to a reduction in growth performance, alteration of blood parameters (erythrocyte/leucocyte count), blood enzymes (Alanine Aminotransferase (ALT), Aspartate transaminase (AST) or Alkaline Phosphatase (ALP)), liver alterations or the suppression of immune parameters. Moreover, it is generally observed that the biological effects of mycotoxins vary greatly over different species, and even in the same species, they depend on the concentration of the toxin in feed, the age of the animal, and its nutritional and health status prior to mycotoxin ingestion (Hendricks, 1994).

The present work aimed to evaluate and elucidate the impact of DON on rainbow trout, by exploring new tools and evaluating new diagnostic factors, which may be used later by the industry as standards to better diagnose mycotoxicoses in fish. Growth performance is one of the most prevalent clinical signs of DON across fish species. Building upon the current knowledge of the impact of DON in rainbow trout (Hooft, Bureau, 2017; Hooft, Elmor et al.,

2011; Matejova et al., 2015; Ryerse et al., 2015), we have attempted to characterise reduced growth performance by exploring the impact of DON on ingredient digestibility. This was achieved by measuring total pepsin, trypsin, chymotrypsin, amylase and lipase activities. The expression level of gene markers for stress regulation (*star*; head kidney; *crf1, crf2, crfbp; brain*), growth control (*igf1, igf2*; liver; *adcyap1a(PACAP)*; brain), digestion, regulation, and appetite control (*sst2, chia, pga, lpl, ghrl, cel1, cel2, cckt, cckn, cckl, amy2a1, atp4a, crtl, try1, try2, try3*; gastrointestinal tract (GIT); *lep, npy*; brain). Moreover, due to DON-related damage of the GIT, faeces was analysed for DON metabolites in order to study the DON metabolism.

2. Material and Methods

2.1. Experimental diets

The trial comprised three dietary treatments (Table 1), all based on a single dietary formulation. The control diet (CTRL) contained a commercially equivalent level of fishmeal (Fishmeal Super Prime = 12.45%; Fishmeal 60 = 14.0%) and moderate levels of plant ingredients such as soy protein concentrate, wheat gluten, corn gluten, wheat meal, soybean meal and corn meal as protein sources. Fish oil was used as the main lipid source. This control diet served also as the base of two additional diets which were supplemented, at the mixing step, with culture material extract containing DON (Romer Labs, Tulln, Austria) at 4.5 and 10.5 mg kg^{-1} (diets DON 5 and DON 11, respectively). Contamination levels were chosen taking into account previous literature (Hooft, Elmor et al., 2011; Matejova et al., 2014; Matejova, Vicenova et al., 2015; Ryerse, Hooft et al., 2015). All diets were isonitrogenous (crude protein, 52.2% dry matter (DM)), isolipidic (17.9% DM) and isoenergetic (gross energy, 22.2 MJ kg^{-1} DM).

2.2. Manufacture of diets

Diets were manufactured by extrusion (pellet size = 2.0 mm) at SPAROS (Portugal) using a pilot-scale BC45 twin-screw extruder (CLEXTRAL, France) with a screw diameter of 55.5 mm; the operating temperature was 113–116 °C. Upon extrusion, feeds were dried in a vibrating fluid DR100 bed dryer (TGC Extrusion, France). Pellets were allowed to cool to room temperature before application of the oil fraction by coating under vacuum conditions (PG-

10VCLAB instrument; DINNISEN, The Netherlands). The target amount of oil for the post-extrusion coating procedure was sprayed onto the pellets under vacuum (760 mbar) for approximately two minutes. The experimental feeds were stored in a cool and aerated location throughout the trial. Samples of each diet were taken for proximate composition analysis (Table 1). A sample of each diet was tested for target mycotoxin presence (DON; Table 1) plus other relevant mycotoxins and metabolites (Table S1). The natural presence of other major mycotoxins, were determined analytically as described previously and reported in table S2.

2.3. Fish and rearing conditions

All procedures involving fish were performed according to the EU guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU).

Quadruplicate groups of 50 rainbow trout (*Oncorhynchus mykiss*), with a mean ± standard deviation (s.d.) initial body mass (IBM) of 2.52 ± 0.03 g, were fed one of the three experimental diets for 60 days. Fish were grown in quadrangular flat-bottom fish tanks (V = 250 L) supplied with well freshwater in a flow-through system; 24.3 ± 0.4 °C water temperature, 8.1 ± 0.7 mg L^{-1} dissolved oxygen, and a 14 hour light : 10 hour dark photoperiod regime. Fish were hand fed to visual satiety three times per day (twice during weekends) with utmost care to avoid feed wastage and allow a precise quantification of feed intake. Furthermore observing feeding behaviour allow us to confirm that the reduced feed intake on DON fed treatments were established progressively after a certain time accepting the feeds, excluding the reduced food intake due to modified organoleptic properties of the feed. Fish were anesthetised with 2-phenoxyethanol (200 mg L^{-1}) for group weighting at the start of the trial (day 0), at day 29, and at day 60.

2.3. Biological sampling

The whole-body proximate composition was analysed from a pool of ten fish sampled and stored (-20 °C) at the beginning of the trial (day 0) and from a pool of three fish per tank sampled and stored (-20 °C) at the end of the trial (day 60). Additionally, at the end of the growth trial, ten fish per tank were anesthetised with 2-phenoxyethanol (200 mg L^{-1}) and a

blood sample was collected by puncturing the caudal vein with a heparinised syringe. Blood samples were centrifuged at 1,590 ×g for ten minutes and the resulting supernatant fraction, i.e. the serum, was transferred to a clean vial, snap-frozen in liquid nitrogen, and stored at -80 °C for the subsequent analysis of metabolites. Immediately after blood collection, fish were euthanised by cervical cut and their livers were dissected and weighed for calculating the hepatosomatic index (HSI). Moreover, samples of stomach, liver, pancreas and intestine (n=10/treatment) were preserved in liquid nitrogen and stored at –80 °C for enzyme activity measurement. Samples of brain, GIT, liver and head kidney (n=10/ treatment) were flash frozen in liquid nitrogen, with the exception of brain samples which were preserved in RNAlater® (Invitrogen Life Technologies) for gene expression analysis. All samples were stored at –80 °C until processed. Sample collection was done randomly three hours after the animals were fed.

2.4. Biochemical composition of feeds, whole fish, and faeces

Analyses of feed, whole fish, and faeces were carried out with analytical duplicates following the methods described by the Association of Official Analytical Chemists (AOAC, 2006). Dry matter was measured after drying at 105 °C for 24 hours. Total ash was analysed by combustion (550 °C for six hours) in an L9/11/B170 muffle furnace (NABERTHERM, Germany). Crude protein (N × 6.25) was analysed by flash combustion followed by gas chromatographic separation and thermal conductivity detection with a Leco N FP-528 analyser (LECO Corporation, MI, USA). Crude lipid was determined by petroleum ether extraction (40–60 °C) using a SOXTEC™ 2055 Fat Extraction System (Denmark). Gross energy was measured in an adiabatic C2000 basic bomb calorimeter (WERKE, Germany), and chromium concentration in feeds and faeces was determined by atomic absorption spectrometry in a SpectrAA 220 FS instrument (VARIAN, CA, USA) (Reis et al., 2008).

2.5. Mycotoxin analyses in feed

The analyses were carried out as described by Streit *et al.,* (2013). All samples were analysed with liquid chromatography tandem mass spectrometry (LC-MS/MS). For the purpose of data analysis, non-detect levels were based on the limit of detection (LOD) of the LC-MS/MS. The LOD for aflatoxin B_1, was 0.3 µg kg^{-1}. For ZEA, DON and OTA, the LODs were 10, 50 and

0.2 µg kg^{-1}, respectively. For FUM the LOD was 25 µg kg^{-1} for FB_1, FB_2, FB_3, and FB_4. The LOD for the remaining toxins/metabolites was 0.5 µg kg^{-1}.

2.6. Apparent digestibility measurements and mycotoxins in fish faeces

At the end of the growth performance trial, and following the sampling procedures described in section 2.3., ten fish per tank were used to determine the apparent digestibility coefficients (ADC) of dry matter, protein, and energy using the indirect method. An inert tracer (0.96% Chromium oxide, Cr_2O_3) was added to the feed and the nutrient to tracer ratio in feed and faeces were used for digestibility measurements. Faeces samples were collected using the apparatus for continuous faeces collection by filtration described by Choubert *et al*., (1979). Over the course of one week, faeces was removed from filters three hours after each feeding and stored at -20 °C. Faeces collected per tank was pooled per treatment and stored at -20 °C for subsequent analysis. Faeces was also analysed for the presence of DON and DON metabolites (DOM-1, DON-3-sulfate and DOM-3-sulfate). Briefly, four 500 mg samples were extracted in duplicate three times from each treatment using 5 ml of 50% methanol. The samples were vortexed and shaken for 30, 20 and 10 minutes. The extraction samples were centrifuged – first and second centrifugations at 3200 rpm for five minutes, and the final centrifugation at 4200 rpm for five minutes - and the supernatants combined, vortexed and centrifuged again at 4200 rpm for five minutes. Prior to LC-MS/MS analysis, samples were diluted in vials (1:1) with the extraction solvent.

2.7 Enzyme Activity Analyses

Enzyme extracts were prepared for enzyme activity measurement from samples previously preserved in liquid nitrogen and stored at -80 °C. Stomach and intestine samples were dissected and homogenised separately. Samples were manually homogenised in 3 mL distilled water and centrifuged for ten minutes at 4 °C at 11,000 rpm (Eppendorf 5810R, Hamburg, Germany). The supernatants from the stomach samples were measured for pepsin activity, and the supernatants from the intestine samples were analysed for trypsin, chymotrypsin, amylase and lipase activities.

Pepsin activity was determined by the method of Anson (1938): 15 µL of extracts were mixed with 1 mL of 0.5% acid-denatured bovine haemoglobin diluted in 0.2 M HCl-Glycine

buffer (pH 3). After incubation at 37°C for 30 minutes, the reaction was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA), cooled to 4 °C for 15 minutes and then centrifuged at 12000 rpm for 15 minutes. The absorbance of the resulting supernatant was measured at 280 nm. Blanks were constructed by adding the enzyme extracts to the reaction mixture just after the TCA. For alkaline protease activities, trypsin activity was assayed using BAPNA (N-benzoyl-DL-arginine-p-nitroanilide) (B4875 Sigma-Aldrich) as a substrate. 0.5 mM BAPNA was dissolved in 1mL dimethyl-sulfoxide (DMSO) and then made up to 100 mL with Tris-HCl 50mM, pH 8.5, containing 20 mM $CaCl_2$. Chymotrypsin activity was determined using 0.2 mM SAPNA (N-succinyl-L-Ala-L-Pro-Phe-p-nitroanilide) dissolved in 1mL DMSO and then made up to 100 mL in the same buffer. Reactions were started in 96-well microplates by the addition of 15 µL of the enzyme extract to 200 µL of the respective substrate and liberation of p-nitroanilline was kinetically followed at 405 nm in a microplate reader (Cytation 3 Cell Imaging Multi-Mode Reader, USA).

Lipase activity was measured following the method described by Versaw *et al.* (1989), with some modifications. The assay mixture contained 60 µL of 100 mM sodium taurocholate, 540 µL of 50 mM Tris-HCl, pH 8.5, 10 µL of enzyme extract and 6 µL of β-Naphthyl caprilate. The reaction was maintained for 25 minutes at 37°C and after this time, 6 µL of 100 mM Fast Blue BB in DMSO was added before being incubated at 37°C for five minutes. The reaction was then stopped with 60 µL TCA 0.72 N. Finally, 815 µL of 1:1 (v:v) ethyl acetate/ethanol solution was added and the absorbance recorded at 540 nm.

Amylase activity was determined by the 3, 5-dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 2% soluble starch as a substrate. 30 µL of enzyme extract and 300 µL of substrate were incubated at 37 °C for 30 minutes. The reaction was stopped by the addition of 150 µL DNS and was heated in boiling water for 10 minutes. Then, after cooling in ice, 1.5 mL of distilled water was added to the mixture and the absorbance was measured at 540 nm. Blanks were constructed by adding the enzyme extracts to the reaction mixture just after DNS.

2.8 Gene Expression Quantification

All the samples were individually processed for total RNA extraction using NucleoSpin® RNA kits (Macherey Nagel). An Ultra-Turrax® T25 with an S25N-8G dispersion tool (IKA®-Werke) was used to homogenise the brain tissue in a volume of homogenising buffer proportional to

the mass of the tissue. The remaining samples (initially frozen in liquid nitrogen) were homogenised in three steps. First, using a mortar and a pestle, then with an Ultra-Turrax® T25 in liquid nitrogen, to finally by taking a sample of less than 30 mg from the finely minced powder to process with an Ultra-Turrax® T10 with the kit homogenising buffer. Genomic DNA (gDNA) was removed via on-column DNase digestion at 37 °C for 30 minutes using rDNase (RNase-free) included in the kit. The RNA concentration was measured with a Qubit 2.0 fluorimeter and Qubit RNA BR assay kit (Life Technologies), whereas RNA quality was checked in a Bioanalyzer 2100 with the RNA 6000 Nano kit (Agilent Technologies). Only samples with an RNA integrity number (RIN) greater than 8.0 were tested using real-time quantitative PCR (qPCR). Total RNA (500 ng) from each sample was reverse-transcribed in a 20 µL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences) in a Mastercycler® proS (Eppendorf) and as previously described by Mata-Sotres *et al.,* (2016). A pool of cDNA from all the samples for each tissue was used for calibration plots, using six serial $1/10^{th}$ dilutions from 10 ng to 100 fg, in order to assess the linearity and efficiency of the different primer combinations, as well as for being used for inter-assay calibration. Control reactions with RNase free water (NTC) and RNA (NRT) were included in the analysis to ensure the absence of primer-dimers and genomic DNA contaminations. The linearity and amplification efficiency for each pair of primers are shown in Table S1. Previously, primers pairs were tested for final working concentrations (optimum 200 nM) and temperature (60 °C). qPCR reactions were performed in triplicate with 1 ng of cDNA (estimated from the input of total RNA) forward and reverse primers (Table S1) for the named samples (200 nM each) and PerfeCTa™ SYBR® Green FastMix™ (Quanta BioSciences). Reactions were performed in a volume of 10 µL using Hard-Shell® Low-Profile Thin-Wall 96-Well Skirted PCR plates (BioRad) covered with Microseal® B Adhesive Seals (BioRad). The thermocycling procedures were carried out with an initial denaturation and polymerase activation at 95 °C for ten minutes, followed by 40 cycles of denaturation for 15 seconds at 95 °C, annealing and extension at 60 °C for 30 seconds, and finishing with a melting curve from 60 °C to 95 °C, increasing by 0.5 °C every five seconds. Melting curves were used to ensure that only a single PCR product was amplified and to verify the absence of primer–dimer artifacts. Relative gene expressions were quantified in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using the $\Delta\Delta C_T$ method (Livak, Schmittgen, 2001), corrected for efficiencies (Pfaffl, 2001), and normalised by geometric averaging of two internal control genes (Vandesompele et al.,

2002), *actb* and *ef1a.* The genes were selected owing to their lower than 0.5 target stability M value and lower than 0.25 CVs (as indicated by BioRad CFX Manager Target Stability Value). A pool of all the cDNA samples was used as a calibrator on every qPCR plate to correct for inter-assay differences. qPCR reactions were carried out with 10 ng of cDNA (assumed from RNA input), forward and reverse primers, and PerfeCTa™ SYBR® Green FastMix™ (Quanta BioSciences) in a final volume of 10 µL. qPCR primer sequences, amplicon sizes, amplification efficiencies, R^2 and GenBank accession number of the sequences are shown in Table S1. RNA nucleotide sequences for *ef1a, actb, star, igf1, igf2, crf1, crf2, crfbp, npy, adcyap1a, lep, sst2, chia, pga, lpl* and *ghrl*, were obtained from GenBank, and nucleotide sequences for *cel, cel2*, *amy2a, atp4a, crtl, try1, try2* and *try3* were retrieved from the *Oncorhynchus mykiss* WGS project database, using Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EMBOSS explorer (http://www.bioinformatics.nl/emboss-explorer/).

2.9 Growth, feed intake, digestibility, and nutrient budget calculations

IBW (g): Initial mean body weight

FBW (g): Final mean body weight

Specific growth rate (SGR; %/day) = (Ln FBW – Ln IBW) × 100/days

Feed conversion ratio (FCR) = crude feed intake / weight gain

Feed intake (FI; %BW / day) = (crude FI / (IBW + FBW) / 2 / days) × 100

Protein efficiency ratio (PER) = wet weight gain / crude protein intake

Hepatosomatic index (HSI) = (liver weight / body weight) × 100

Nutrient retention (%) = ((FBW × NFF) – (IBW × NIF))/Nutrient intake × 100,

with NFF being the nutrient content of final fish and NIF the nutrient content of initial fish.

Apparent digestibility coefficient (ADC, %) = 100 × [1 – (% Cr_2O_3 feed / Cr_2O_3 faeces) × (% nutrient faeces / % nutrient feed)]

Daily nitrogen (N) gain = ((final body N content – initial body N content) / (IBW+FBW))/ 2 / days

Daily N intake = (N intake / (IBW + FBW) / 2) / days

Daily faecal N losses = daily N intake × (100 – ADC Protein) / 100

Daily metabolic N losses = daily N intake – (daily N gain + daily faecal N losses)

2.10 Statistical analysis

One-way analysis of variance (ANOVA) was used to compare differences between the three diets. A post-hoc Tukey honest significant difference (HSD) test was used when ANOVA results revealed significant differences ($P < 0.05$). The homogeneity of variances was previously tested using Levene's test, and all parameters expressed as percentages were subjected to arcsin square root transformation. Data are presented as the mean of three replicates ± s.d. All statistical tests were performed in IBM SPSS Statistics 18 software (IBM Corp., USA).

3. Results

3.1. Zootechnical performance

3.1.1. After 29 days of experimental feeding

The survival rate of the rainbow trout was high (>97.0%), across the three dietary treatments with only minor mortality in the DON 5 and DON 11 treatments, that was not significant ($P<0.05$; Table 2). After 29 days, all other zootechnical parameters (FBW, g; SGR, %/d; FCR; FI, %; ABW/d; PER), were significantly affected by dietary inclusion of DON ($P<0.001$; Table 2). FBW ranged from 3.90 to 9.39 g. Fish fed the CTRL diet showed a significantly higher FBW, SGR, FI and PER than those fed both DON supplemented diets (DON 5 and DON 11) ($P<0.001$). Additionally, fish fed the highest DON dose (DON 11) showed a significantly lower FBW, SGR, FI and PER than those fed with the DON 5 diet ($P<0.001$). The FCR varied between 0.96 and 1.56. Fish fed the CTRL and DON 5 diets showed a significantly lower FCR than those fed the DON 11 diet ($P<0.001$).

3.1.2. After 60 days of experimental feeding

After the second experimental period, mortality was observed in all treatments (Table 3). The survival rate of the fish fed CTRL and DON 5 diets was significantly higher than that observed in fish fed the DON 11 diet ($P=0.02$). Fish from the best performing treatment (CTRL) had a 9.8-fold increase of their initial body mass, with FBW ranging from 5.96 to 24.77 g. Fish fed the CTRL diet showed a significantly higher FBW, SGR and FI than those fed both DON supplemented diets (DON 5 and DON 11; $P<0.001$). Additionally, fish fed the highest

DON dose (DON 11) showed a significantly lower FBW, SGR and FI than those fed with the DON 5 diet ($P<0.001$). Fish fed the CTRL and DON 5 diets showed a significantly lower FCR than those fed the DON 11 diet ($P=0.001$). The HSI varied between 1.62 and 2.47, with CTRL fish showing a significantly lower HSI than those fed both DON supplemented diets (DON 5 and DON 11; $P<0.001$; Table 3).

3.2 Whole-body composition

The whole-body composition of fish in terms of moisture, fat and energy was not affected by the various dietary treatments ($P>0.05$, Table 4). However, fish fed the CTRL diet showed a significantly lower whole-body ash and a significantly higher whole-body protein than those fed both DON supplemented diets (DON 5 and DON 11; $P<0.001$).

3.3 Whole-body nutrient retention and apparent digestibility

Protein retention varied between 17.83 and 32.56%, and fish fed the CTRL diet showed a significantly higher protein retention than those fed both DON supplemented diets (DON 5 and DON 11) ($P<0.001$; Table 5). Moreover, fish fed the highest DON dose (DON 11) showed a significantly lower protein retention than those fed with the DON 5 diet ($P=0.001$). Fat retention ranged from 58.98 to 81.86%, while energy retention varied between 31.09 and 40.16%. Fish fed the CTRL and DON 5 diets showed a significantly higher fat and energy retention than those fed the DON 11 diet ($P<0.001$; Table 5). Digestibility of dry matter, protein and energy were not affected by dietary treatments ($P>0.05$; Table 6).

From DON and DON metabolites (DOM-1, DON-3-sulfate and DOM-3-sulfate) analysed in trout's faeces, only DON and DON-3-sulfate were detected (Figure 1). No DON or DON metabolites were detected in animals fed the control diet (CRTL). From the total ingested DON in feed, 13.2% was recovered in faeces from treatment DON 5 and 10.5% from DON 11. The high solubility of DON in water may explain such low recovery rates. From the recovered mycotoxin in faeces, more than 80% of it was in the form of DON-3-sulfate (DON 5 = 84.07% and DON 11 = 82.09% of DON-3-sulfate) and around of 15% of DON (DON 5 = 15.93% and DON 11 = 17.91% of DON).

3.4 Enzyme Activity Analyses

The results of total pepsin, trypsin, chymotrypsin, amylase and lipase activities measured in the different experimental groups are shown in Figure 2 (i to v, respectively). For total pepsin, DON 11 showed a higher activity (0.0021 ± 0.0003 U g^{-1} BW; P<0.001) when compared to the CTRL (0.0012 ± 0.0002 U g^{-1} BW) and DON 5 (0.0013 ± 0.0003 U g^{-1} BW) groups. In contrast, trypsin, showed its lowest activity value in the DON 5 group (2.09 ± 1.25 U g^{-1} BW; P=0.043) when comparing to DON 11 (2.40 ± 1.19 U g^{-1} BW), however, statistically similar (P>0.05) to the control (2.58 ± 2.08 U g^{-1} BW) and DON groups. Chymotrypsin did not present significant differences (P>0.05) among the different experimental groups. DON 11 presented a higher lipase activity (0.0022 ± 0.0001 U g^{-1} BW; P=0.002) compared to CTRL (0.0005 ± 0.00001 U g^{-1} BW) and DON 5 (0.0013 ± 0.0007 U g^{-1} BW). Amylase did not present significant differences (P>0.05) among the different dietary groups.

3.5 Gene Expression

The relative expression levels of gene transcripts for stress regulation (*star*; head kidney; *crf1, crf2, crfbp;* brain), growth control (*igf1, igf2*; liver; *adcyap1a*; brain), enzymatic digestion, regulation and appetite control (*sst2, chia, pga, lpl, ghrl, cel1, cel2, cckt, cckn, cckl, amy2a1, atp4a, crtl, try1, try2, try3*; GIT; *lep, npy*; brain) are shown in Table 7. Expression of *igf1* and *igf2* was significantly lower in DON 5 and DON 11 when compared to CTRL group (P=0.004 and P=0.008, respectively). Interestingly, expression levels of *npy* and *adcyap1a/PACAP* mRNAs were significantly up-regulated by DON treatments (P=0.004 and P=0.005, respectively). In contrast *star* mRNA transcripts displayed a trend toward higher abundance in both DON fed treatments, however this was not significant (P=0.088). Analyses of specific mRNA transcript levels across all enzyme precursors (*crtl, Try1, Try2, Try3)*, highlighted *try3* to be significantly up-regulated (P=0.036) in both DON fed groups.

4. Discussion

The impact of DON on fish has been further elucidated in recent years with the rainbow trout as a useful model. However, very little is known in comparison to land-farmed animals. This is especially true regarding diagnostic parameters to correctly identify the impact of DON ingestion in a production setting. For this purpose, an experimental protocol was

designed to further understand the impact of DON on growth performance and also to explore the underpinning causes for the reported decreases in growth performance. The DON effect was evaluated by studying the regulation of digestion, both at enzyme activity level and mRNA gene expression, where we surveyed stress regulation, growth and appetite control processes. In order to investigate metabolic breakdown of DON faeces was analysed for DON metabolites.

Growth performance was affected by DON (4,714 ± 566 and 11,412 ± 1,141 μg/kg), in a similar manner to that previously described (Hooft, Elmor et al., 2011; Matejova, Vicenova et al., 2015; Ryerse, Hooft et al., 2015), despite higher DON concentrations. Fish fed DON showed a significantly lower FBW, SGR and FI when compared to the control ($P<0.001$). Moreover, the highest DON dose (DON 11) showed a significantly lower FBW, SGR and FI than the dose used in the DON 5 diet ($P<0.001$). In addition, FCR was significantly increased in fish fed the DON 11 diet ($P=0.001$). The HSI was significantly higher in animals fed DON, although this difference was not observed during dissection or visual examination for clinical signs. Interestingly, no clinical signs (e.g. internal or external haemorrhages, dermal and oral lesions, abnormal pigmentation or damage to fins) were detected on animals fed DON, confirming that diagnosis of DON ingestion is extremely difficult, even at high dosages (DON 11; 11,412 ± 1,141 μg/kg). According to previous studies, the impact of DON might vary greatly depending on unknown factors, even for the same species. For the same range of DON contamination (0.3 to 5.9 ppm), some authors (Hooft, Elmor et al., 2011; Ryerse, Hooft et al., 2015) did not find any major pathological changes in the distal intestine of trout, while in other situations, gastrointestinal haemorrhages were found (Matejova, Modra et al., 2014). Despite the lower growth in the DON fed treatments, which was visually detected (Figure S1), DON 11 treated fish also had significantly lower survival rates. In our opinion, it is highly unlikely under production conditions that such an increase in mortality/decrease in performance would be associated to DON ingestion particularly when specific clinical signs are lacking. The present scenario illustrating the significant impact of DON in trout coupled to a complete lack of clinical symptoms highlights the need for further investigation to support an early diagnosis for DON ingestion.

Anti-nutritional factors (ANFs) that decrease enzymatic activity, or form complexes with proteins thereby modifying digestion processes have been described (Santigosa et al., 2008) (Moyano et al., 1999). However, very little is known about the impact of mycotoxins on

digestive enzymes and information regarding the impact of DON is very scarce. For AF ingestion, Han *et al.* (2008) observed increased protease, amylase, chymotrypsin, and trypsin activity and an apparent decrease in digestibility of crude protein in 42-day old ducks fed 0.02 and 0.04 mg kg^{-1} AF. To our knowledge most studies have been focused on the effects of DON on the nutrient absorption process (Grenier, Applegate, 2013), however there is no information available regarding the effects of DON on digestive enzymes.

The major contributing factor to the conversion of feed to growth is protein turnover thus proteases play an essential role. Proteolytic enzyme activity (pepsin, trypsin and chymotrypsin) was significantly altered in DON fed groups. Total pepsin activity was significantly higher in the DON 11 group (0.0021 ± 0.0003 U g^{-1} BW; $P<0.001$) compared to the CTRL (0.0012 ± 0.0002 U g^{-1} BW) and DON 5 (0.0013 ± 0.0003 U g^{-1} BW) groups. However, the observed impact upon pepsin might be directly related to the decrease of feed intake in this group, and not necessarily a direct impact of DON on pepsin or any pepsin precursor. It is well described that pepsinogen is rapidly synthesised during feeding and then secreted, whereupon pepsin activity increases. For example, Einarsson *et al.,* (1996) observed that under starvation conditions in *Salmo salar* there was a slight rise in pepsin activity in the stomach mucosa suggesting that pepsin can be stored in salmonids. Therefore, and taking into account that animals were fed three hours prior to sampling, we cannot exclude the hypothesis that higher pepsin secretion in the DON 11 group could be related to lower feed intake. This in turn may result in a retention of pepsin in stomach mucus, due to a markedly reduced stomach evacuation. Chymotrypsin did not show significant differences between treatments, although a numerically lower activity was observed in the DON 11 group. Rungruangsak-Torrisen *et al.* (2006) observed a higher specific activity of chymotrypsin when growth was limited or depressed due to starvation or food deprivation. However, considering that feed intake was reduced in the DON 11 group, possibly due to endogenous reasons, no major effect was observed in chymotrypsin activity. As trypsin activates chymotrypsin in fish (Sunde et al., 2001) it is difficult to find out if DON impacted, directly or indirectly, this activation. Trypsin showed its lowest activity value in the DON 5 group (2.09 ± 1.25 U g^{-1} BW; $P=0.043$), but this was statistically similar to the CTRL (2.58 ± 2.08 U g^{-1} BW) and DON 11 (2.40 ± 1.19 U g^{-1} BW) groups ($P>0.05$). However, due to the natural variation and lack of resolution for the enzyme activities, is very difficult to identify specific changes. Also interesting is the observation that from the four types of alkaline

proteases (*ctrl*; *try1*, *try2* and *try3*), only mesotrypsinogen (trypsinogen-3) showed a higher expression level in DON fed treatments. Mesotrypsin is a specialised protease known for its resistance to trypsin inhibitors. It is thought to play a special role in the degradation of trypsin inhibitors, possibly to help with the digestion of inhibitor-rich plant meals such as soybeans and lima beans, which might be the case of plant meals containing DON as well (Szmola et al., 2003), however more research is needed on this topic.

Both trypsin activity and mRNA expression levels for *igf1* and *igf2* were found to be significantly higher in the CTRL. It is well known that trypsin cleaves protein at the carboxyl side of the basic amino acids lysine and arginine (Stryer, 1988), which elevates plasma insulin levels in salmonids (Plisetskaya et al., 1991). In turn, insulin stimulates amino acid uptake and protein synthesis especially in the muscle tissue (Matty, 1986; Murat et al., 1981), leading to a growth promoting effect in salmonids (Donaldson et al., 1979). Proteolytic enzyme differences observed between the treatments were probably not associated with secretion control, as cholecystokinin-like peptides (*cck-t; cck-n or cck-l*) did not significantly alter due to DON treatment.

Apparent digestibility of dry matter, protein and energy in the present study was not affected by dietary levels of DON. In current literature, contradictory information about the effects of DON on ADC has been reported. While, for example, Dänicke *et al*. (2004) and Van Le Thanh *et al.* (2015) reported that DON could affect crude protein digestibility in piglets, Jo *et al.* (2016) reported no differences in ADC in growing pigs fed 10,000 µg kg^{-1} DON. The latter is in agreement with findings from the present study for similar levels of contamination. Interestingly, Jo *et al.* (2016) stated that DON contamination might affect essential amino acid digestibility, DON may affect trypsin (confirmed in this study), and trypsin will influence the levels of insulin, which will ultimately influence amino acid uptake however this requires further research to verify whether this pathway is directly impacted by DON. In this study protein, fat and energy retention were all significantly affected in animals fed DON. The low performance of the animals fed DON could be a consequence of decreased nutrient uptake and transport rather than lower nutrient digestibility, as enzyme activity and ADC appear unaffected by DON. The reason behind the enzyme activity differences among the experimental groups is not clear, however differential feed intake may influence our interpretation.

The neuroendocrine process that controls satiety is regulated, with others, by Neuropeptide Y, Leptin, Ghrelin or Adenylate cyclase-activating polypeptide (PACAP). In the present work, leptin and ghrelin mRNA transcripts were not influenced by DON which was not surprising as Leptin activity is related to long-term regulation of energy balance, suppressing food intake, while Ghrelin is a fast-acting hormone acting as "stopper" after meal initiation (Klok et al., 2007). In contrast, PACAP plays an important and direct role in the regulation of feed intake. In goldfish, it has been observed that intracerebroventricular injections of PACAP suppress food intake (Matsuda et al., 2005). In the present study, upregulation of *adcyap1a* or PACAP mRNAs provides a possible link to the observed reduction in feed intake, as described in the literature (Chance et al., 1995; Li et al., 2015; Morley et al., 1992; Mounien et al., 2008; Tachibana et al., 2003). In zebrafish, PACAP greatly decreases the frequency of gut motility waves (Holmberg et al., 2004) which might also have an impact on nutrient absorption. *npy* was also upregulated in DON fed treatments, however its putative role in our experimental setup is challenging to explain. In mammals, *npy* is a key factor in the regulation of feeding behaviour and there is strong evidence of a direct physiological role of *npy* and its expression levels in controlling feed intake (Chamorro et al., 2002). However, most of the studies published suggest that elevated brain *npy* levels induce increased feed intake (see review Chamorro et al., 2002), which is contrary to the obtained results in this study. Though, in the present study the upregulation of *npy*, seems to be a consequence of the reduce feed intake, i.e., *npy*, as explained by (Narnaware, Peter, 2001), is regulated in part by the feeding state of the animal, since food deprivation induces a marked increase in both *npy* and its mRNA levels in the brain. Narnaware and Peter, 2001, observed an increased *npy* mRNA expression in several brain regions of goldfish in response to food deprivation, which might help to explain the findings of the present study.

Metabolisation of DON could also explain the lack of any lesions in trout when compared, for example, with swine and poultry. Metabolisation of trichothecenes in several livestock species has been reported however, these studies focus on the formation of de-epoxy-DON or on glucuronidation (Dänicke, Valenta et al., 2004; Eriksen et al., 2002; Schwartz-Zimmermann et al., 2015). While de-epoxy-DON is achieved mainly by gut microbiota, glucuronidation is carried out by endogenous UDP-glucuronosyltransferases in the liver, and possibly also in intestinal microsomes (Maul et al., 2015). Metabolisation pathways of DON vary greatly within species (Schwartz-Zimmermann, Fruhmann et al., 2015). In fish, only one

report in brown bullhead catfish (*Ameriurus nebulosus*) has shown the capability of the gut microbiota of this species to biotransform trichothecenes into their de-epoxy forms (Guan et al., 2009). DON can also be metabolised by sulfation, which was only recently discovered as a major pathway for chickens and turkeys (Devreese et al., 2015; Schwartz-Zimmermann, Fruhmann et al., 2015; Wan et al., 2014). In the present study, it has been shown for the first time in rainbow trout that DON is metabolised into DON-3-sulfate, which is less toxic than DON. Despite considerable mycotoxin leaching from the faeces, due to the high solubility of DON/DON-metabolites in water, more than 80% of the mycotoxin in faeces was recovered in the form of DON-3-sulfate. The location of formation, absorption and elimination of DON-3-sulfate is not known and was not further investigated in the present trial. However, as suggested by (Schwartz-Zimmermann, Fruhmann et al., 2015), DON might be metabolised to DON-3-sulfate in the intestinal mucosa, liver, or even in the kidney as happens for some other vertebrate species. The formation of DON-3-sulfate, could also explain the absence of major clinical signs in trout fed DON, particularly at DON 5 treatment levels, which still had a considerable impact on feed intake but did not cause major lesions.

In the present work, the impact of DON on the GIT or on the absorptive process was not evaluated. The potential impact of mycotoxins on the GIT in livestock species is well described (see reviews; (Broom, 2015; Grenier, Applegate, 2013). Due to the mode of action of DON (as an inhibitor of protein synthesis) and the high rate of protein turnover in intestinal cells, it is to some extent also expected to observe altered intestinal areas in trout. However, the literature is not consistent when reporting the impact of DON on the trout GIT (Hooft, Elmor et al., 2011; Matejova, Modra et al., 2014; Ryerse, Hooft et al., 2015). Despite only being evaluated macroscopically, any potential microscopic intestinal damage caused by DON and the consequent influence on nutrient absorption cannot be discarded. However, the novel fact that 80% of DON is metabolised into DON-3-sulfate might help to explain the lack of consistency in GIT damage in trout. The elucidation of metabolisation pathways in fish in respect to DON would be a major step toward understanding the underpinning mechanisms of sensitivity/resistance to this mycotoxin in fish.

5. Conclusion

Deoxynivalenol exposure in fish has been characterised mainly by reduced feed intake and growth performance. Contrary to land farm animals, DON ingestion in fish does not lead to specific clinical signs, except anorexia (at high dosages, above 5 ppm), and some minor altered blood parameters (blood parameters (erythrocyte/leucocyte count), blood enzymes (ALT, AST or ALP), liver alterations or immune parameters suppression) which are generally not specific for DON-induced mycotoxicosis. In the present study, it was observed that digestive enzymes (regarding activity and mRNA expression) are affected, however we were unable to clarify if this was caused by DON ingestion or by suppression of feed intake. Moreover, nutrient (protein, fat and energy) retention was affected by dietary DON suggesting that nutrient uptake and transport might be affected. Upregulation of PACAP seems to be fundamental for explaining the reduction of feed intake in DON fed treatments, inducing anorexia. Further research is needed focusing on the effect of DON on appetite control by addressing the influence of DON on the hypothalamic melanocortin system. Suppression of appetite due to DON contamination in feeds might be a defence mechanism in order to decrease the exposure of the animal to DON, therefore reducing the potential negative impacts of DON. The discovery of DON-3-sulfate as a novel trout metabolite makes it a potential biomarker of DON exposure. However, further characterisation of its toxicological relevance is essential.

6. Acknowledgments

The authors would like to thank Marina Machado, Filipa Fontinha, Paulo Santos, Sergio Fernández-Boo, Lourenço Ramos-Pinto and Bruno Reis from the Interdisciplinary Centre of Marine and Environmental Research of the University of Porto for their help on final sampling. Many thanks to Caroline Noonan for the manuscript proofreading. The authors would also like to thank the support of WiseFeed project (European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 691150).

Contribution

The experimental design was from the responsibility of RAG, DS, GMR and SM. *In vivo* experiment and digestibility measurement was at the responsibility of JD. Enzyme activities were performed by CN, MY and FJM. Gene expression were performed by RAG, NG, GMR. Mycotoxin metabolites were analysed by GB and TC. Results analyse, writing, statistical treatment and interpretation of the data, was the responsibility of RAG with the active collaboration of all co-authors. All authors have approved the final article and contributed for its revision.

Declaration of interest

The present work was performed under the WiseFeed project (European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 691150) being Biomin Holding GmbH, a partner of the project, represented by Rui A. Gonçalves.

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